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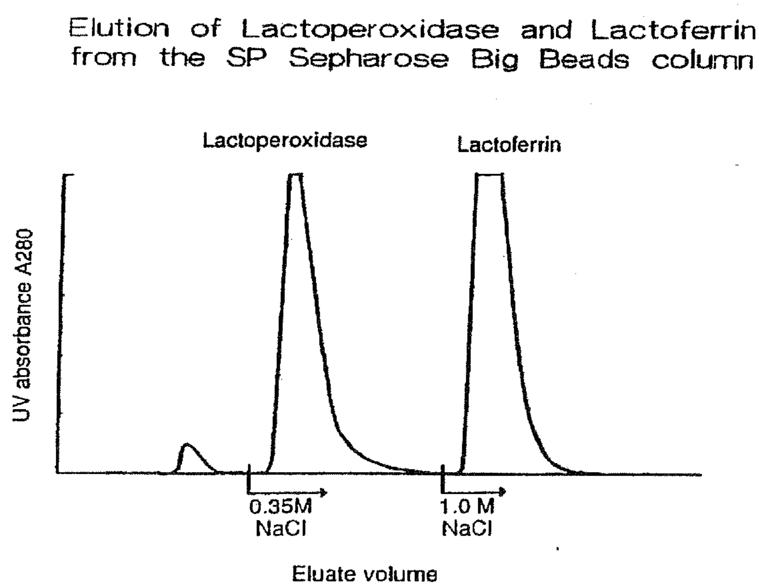
(54) Title: PROCESS FOR ISOLATING LACTOFERRIN AND LACTOPEROXIDASE FROM MILK AND MILK PRODUCTS, AND PRODUCTS OBTAINED BY SUCH PROCESS



(57) Abstract

The invention relates to a process for isolating bioactive proteins such as lactoperoxidase and lactoferrin from milk and/or milk derivatives, characterized by the steps of: a) adsorbing said proteins to a cation exchanger by passing the milk or the milk derivative over the cation exchanger at a high superficial velocity (more than 500 cm per hour) and a high liquid load (100-600 bed volumes per hour); b) eluting these proteins, separately or simultaneously, by elution with one or more salt solutions, so as to form one or more eluates, optionally followed by c) drying of the said eluates. The invention further relates to products obtained by this process.

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Title: Process for isolating lactoferrin and lactoperoxidase from milk and milk products, and products obtained by such process.

The present invention relates to a process for isolating the metalloprotein lactoferrin and the enzyme lactoperoxidase from milk and milk products on an industrial scale.

Lactoferrin (LF), a metalloprotein, occurs in minor amounts *inter alia* in milk from mammals. It is capable of binding 2 mol of iron per mol of protein. It thus provides the young mammal with iron in assimilable form. It moreover functions as a bacteria inhibiting agent for pathogenic microorganisms that are iron-dependent.

The enzyme lactoperoxidase (LP) catalyzes the breakdown of hydrogen peroxide in the presence of a hydrogen donor or an oxidizable substance. In milk, it functions as a bacteria inhibitor, *inter alia* of pathogenic Streptococci and Salmonella in the presence of thiocyanate and peroxide, both present in milk. In raw cow's milk, concentrations of about 200 mg/l have been reported for lactoferrin and concentrations of about 30 mg/l for lactoperoxidase, depending *inter alia* on the stage of lactation and breed.

Lactoperoxidase in particular is thermolabile. Upon pasteurization, this enzyme will be inactivated in part or in whole, depending on the intensity of the heat treatment. It is therefore obvious to start from raw milk for the isolation of these two components. However, this is technologically less interesting, because small amounts of LP and LF must be separated from large amounts of other substances (proteins, sugars, fats). Moreover, it is uncertain whether the milk from which LP or LF has been withdrawn can still be used as "milk" in terms of the official food regulations, for human

consumption and/or as raw material for making cheese and butter.

It is therefore obvious not to start from raw milk, but, instead, to start from a by-product such as formed in cheese or butter making, namely, whey or skim milk. In cheese making, the milk is subjected only to a minor heat treatment. As a consequence, substantially all of the lactoperoxidase is still present in cheese whey. However, only a part of the lactoferrin remains present, namely, about 25%. Nevertheless, whey is an attractive starting material. It is available in large amounts, cheap, and, in a manner of speaking, pre-purified.

NL-A 8201947 discloses a process for isolating LP and LF from cheese whey. Whey having a pH between 7.5 and 8.2 is applied to a column filled with buffered silica gel. The proteins adsorbed to the column can be eluted, whereby a lactoferrin preparation and a lactoperoxidase preparation are obtained. The purity of the preparations obtained is low, namely, only 66%.

EP-A 0 253 395 discloses the isolation of lactoferrin alone from raw milk and whey. Milk or whey is brought in contact with a weakly acid ion exchanger with carboxymethyl groups as active groups. Various types of exchangers were compared and characterized by a binding capacity for hemoglobin. The examples describe long contact and elution times. The experiments were performed both columnwise and batchwise. This implies that the process described in EP-A 0 253 395 is less suitable for large-scale industrial production. Moreover, only lactoferrin is isolated, whilst lactoperoxidase is not.

EP-A 418 704 discloses the extraction of lactoperoxidase and lactoferrin via affinity chromatography in a column with sulfonated polysaccharide resin. LP and LF are adsorbed and subsequently eluted and purified. In the examples described, a small liquid load is used (about 80 bed volumes per hour), yielding good separation of LP and LF.

WO-A 89/04608 discloses a process whereby, starting from cheese whey, LP and LF could be isolated on a semi-industrial scale. Here, too, the rate of throughput of 1-1.5 bed volume per minute is a limiting factor, in view of the large volumes of cheese whey that must be passed through the columns.

The above-mentioned processes are based on known chromatographic techniques for the extraction and purification of proteins from a matrix containing many attendant substances, such as carbohydrates, fats and salts. The above-mentioned techniques are used in particular on a laboratory scale and also on a semi-industrial scale. The resin and ion exchangers that are typically used in these techniques are characterized in particular by a selective binding of the desired proteins/protein fractions, optimum binding capacity and the best possible resolution. This implies that very fine particles (about 100 μm) are used, which enable optimum operation in terms of selectivity, binding capacity and yield. A disadvantage of fine-grained column packings is their high flow resistance. This implies that only low superficial velocities can be used (for instance, up to 500 cm per hour). This does not present a problem in analytical and/or preparatory processes for isolating proteins, particularly not if the concentration of the starting materials is sufficiently high. When the concentrations are very low, as with LF and LP in whey, large quantities of liquid must be passed through the columns. If the superficial velocity is increased, the pressure on the column and the column material will increase. The maximum pressure the ion exchanger itself is capable of resisting, limits the superficial velocity. The pressure, or, better, the pressure drop, is usually expressed in bar per meter bed height. Accordingly, the bed height becomes the limiting factor. This implies that the diameter of the column must be chosen to be large if large amounts of liquid are to be processed per unit time (high flow rates). This requires that the construction of the column has to meet extremely high standards in order to provide the required mechanical robustness and adequate distribution of the liquid. This has

consequences for the economy of the process, on the one hand on account of the cost of such columns, and, on the other, on account of the high cost of the resin.

A process has now been found which makes it possible to isolate the bio-active proteins LP and LF from milk or a milk derivative on an industrial scale by:

a) adsorbing these proteins to a cation exchanger by passing the milk or the milk derivative over the cation exchanger at a high superficial velocity (more than 500 cm per hour) and at a high liquid load (100-600 bed volumes per hour);

b) eluting these proteins, separately or simultaneously, by elution with one or more salt solutions, so as to form one or more eluates, optionally followed by

c) drying of the said eluates.

In the preferred embodiment, the starting material is cheese whey (obtained in cheese making) or neutralized casein whey (pH 6.5) obtained from skimmed milk (after casein precipitation). One condition is that the heat treatment in preceding processes is so mild that sufficient amounts of LP and LF are still present.

Cheese whey which has been obtained in the conventional manner in cheese making is suitable. Because the process as a whole takes a relatively long time and the cheese whey contains microorganisms as a result of the cheese preparation, it is recommended to reduce the number of microorganisms in this phase. For the reasons mentioned above, the conventional heat pasteurization does not come into consideration, but bactofugation, ultrafiltration over a coarse filter or microfiltration do. An additional advantage involved here is that coarse contaminations and protein- and fat-agglomerates are removed at the same time. This whey is subsequently passed at a high velocity over a coarse-grained ion exchanger which also comprises a large number of functional groups.

The cation exchanger used in accordance with the present invention preferably has a mean (number) particle diameter of at least 100 µm, preferably at least 125 µm, more in particular

at least 150 μm. The upper limit for the particle diameter is preferably 300 μm as higher diameters are not expected to provide additional advantages. Use of such particle sizes provides the possibility to have high superficial velocities in the adsorption.

Additionally it is preferred that the cation exchanger has such a high physico-mechanical stability, that a pressure drop exceeding the usual values of 10 bar/m bed height, such as 40 bar/m bed height, can be applied.

It was found that an exchanger such as the SP Sepharose Big Beads (Pharmacia) meets these criteria and accordingly functions eminently.

The mechanical stability of such an ion exchanger allows a pressure drop of at least 40 bar/m bed height. The use of a working pressure of up to 3 bar/10 cm bed height resulted in superficial velocities of 3,000 cm/hour, and rates of throughput of up to 600 bed volumes per hour. In a preferred embodiment the cation exchanger is present as an adsorption bed in a column, the superficial velocity of the milk and/or milk derivatives being 2,000-3,000 cm per hour and a liquid load of 100 to 300 bed volumes per hour.

In addition to a high rate of flow the economy of the process is also determined by the binding capacity and binding rate of the resin. It has now been found that the resin used could be charged to, respectively, about 60 g lactoferrin and 30 g lactoperoxidase per liter of resin. This implies that the active groups of the resin, even at such high rates of flow, are utilised substantially completely. It was also found that, even at the high rate involved, the LP and LF were substantially completely adsorbed to the column. Only when the above-mentioned binding capacity has been closely reached more leakage occurs (more than 20%). According to a preferred embodiment the binding capacity of the ion exchanger is more than 10 g lactoperoxidase and more than 10 g lactoferrin per liter bed volume and more than 80% of the said proteins is extracted.

The ion exchanger is preferably priorly conditioned with a phosphate buffer of pH 6.5 (0.025 mol Na_2HPO_4/NaH_2PO_4). A throughput of about 4 bed volumes of buffer is sufficient to equilibrate the column. After charging the column with clarified cheese whey, the column is first rinsed with buffer to displace the last cheese whey residues. The lactoferrin and lactoperoxidase can be eluted from the column either separately or simultaneously.

If elution is effected with a concentrated buffered salt solution (>0.7 molar NaCl), LP and LF are eluted simultaneously. Elution with a low salt concentration (about 0.2-0.5 molar NaCl) only yields lactoperoxidase. A subsequent elution with a higher salt concentration (up to 2.5 molar NaCl) then yields only lactoferrin. In this way, these two bio-active proteins can be eluted separately.

These eluates are not suitable to be used as such, because the salt concentration is too high. For desalting, the conventional techniques such as ion exchange, electrodialysis, and ultrafiltration/diafiltration are eligible. The last-mentioned technique is preferred because in that manner any desired residual salt concentration can be readily set. The LP and LF fractions, thus unsalted or not, can subsequently be dried. Conventional techniques such as spray-drying, vacuum-drying, roller-drying and freeze-drying are eligible. In view of the thermolability of these bio-active proteins, freeze-drying is the most important and preferred option among them, but the other techniques are certainly eligible as well, if the proper precautions with regard to the heat load are taken.

The invention is further explained and illustrated in and by the following non-limiting examples.

Example 1

An ion exchange chromatography column having a diameter of 1.6 cm was packed with 20 ml of the strong cation exchanger S Sepharose Big Beads (Pharmacia). The bed height was 10 cm. Clarified cheese whey, obtained by cross-flow microfiltration

1.4 µm (Alfa Laval), having a solids content of 5.6% and a pH of 6.6, was pumped through the column at different superficial velocities (cm/hour) at room temperature, until conditions were stable (about 10 minutes). The pressure drop (delta P) across the bed of the ion exchanger in the column was measured as a function of the superficial velocity. A similar series of experiments was carried out with the same column packed with the strong cation exchanger S-Sepharose Fast Flow (Pharmacia).

It was found that at pressure differences in excess of about 1.5 bar, the S Sepharose Fast Flow column bed was increasingly compressed. The loss of porosity as a result of this physicomechanical instability gave rise to a further increase of the pressure drop, which rendered this resin unsuitable for use. The supplier of this resin specifies a liquid velocity of up to 400 cm/hour at a pressure drop of 1 bar/15 cm. The measuring results are summarized in the following table:

Superficial velocity cheese whey in cm/hour	bed volumes/hour	Pressure drop across the column bed in bar S Sepharose BB	S Sepharose FF
600	60	< 0.1	0.5
900	90	0.25	1.2
1,200	120	0.5	2.0*
1,800	180	1.2	7.0*
2,400	240	1.9	-
3.000	300	2.5	-
3,600	360	3.2	-
4,500	450	4.0	-

* stable conditions not possible

The S Sepharose BB column keeps its physical and mechanical stability and thus its excellent performance characteristics to a pressure drop of at least 4 bar/10 cm bed height. This

means that the column can resist a superficial velocity of at least 4500 cm/h.

Example 2

In this Example, it was determined whether the data from Example 1 also apply on a larger scale.

A column of a pilot chromatography system, with a diameter of 10 cm, was packed with the ion exchanger SP Sepharose Big Beads. The bed height was 11 cm and the bed volume was 0.864 l. Clarified cheese whey having a solids content of 5.6% and a pH of 6.6 was pumped through the column at different superficial velocities (cm/hour) at room temperature and the pressure drop across the column bed was measured as a function of the superficial velocity. The superficial velocity was varied within the range of 300 to 2,800 cm/hour, corresponding with 27 to 255 bed volumes/hour.

A similar series of experiments was carried out with the same column filled with the ion exchanger S Sepharose Fast Flow, with variation of the superficial velocity within the range of 200 to 880 cm/hour, corresponding with 18 to 80 bed volumes/hour.

The measuring results are shown in the graph of Fig. 1, where the pressure drop (delta P) across the column bed is plotted as a function of the superficial velocity. It appeared that at a pressure drop in excess of 1.5 bar, the S Sepharose Fast Flow column bed was appreciably compressed. It was further established that at a set pressure drop from about 1 bar, the superficial velocity decreases over time, so that, after some time, the column became unsuitable for continuous use.

The column packed with SP Sepharose Big Beads maintains its good through-flow properties and physical stability at least up to the maximum superficial velocity and pressure drop applied.

Figure 1 shows *inter alia* that, at a pressure drop of 1 bar, the superficial velocity (and hence the number of bed volumes/hour) is about three times higher with the SP

Sepharose Big Beads than with the S Sepharose Fast Flow. Moreover, when the SP Sepharose Big Beads column is used, a much higher pressure drop can be realized, so that the advantage as to the superficial velocity (cm/hour) or bed volumes/hour relative to the S Sepharose Fast Flow column is even considerably higher still, for instance by a factor of 5 or 6 at a pressure drop of 2.5 bar across the column bed of SP Sepharose Big Beads.

In the graph of Fig. 2, the results of the SP Sepharose Big Beads column are compared with those of Example 1.

Example 3

The particular economic and technological advantages of the column-process for the selective adsorption of LP and LF according to the invention, in particular with respect to dimensioning and use on an industrial scale, are illustrated by a comparison of situations with a conventional cation exchanger on the one hand and an improved type of cation exchanger with reduced flow resistance on the other.

As an example of dimensioning on an industrial scale, the starting point is a cheese whey load on the column of $20 m^3$/hour, being the output of a Dutch cheese factory of typical size.

Conditions for the comparison:

Conventional cation exchanger: S-Sepharose Fast Flow. The pressure drop across a packed column when charged with cheese whey is maximally about 6.7 bar/m bed height, at a superficial velocity of maximally 400 cm/hour.

Improved cation exchanger: S-Sepharose Big Beads. The pressure drop across a packed column when charged with cheese whey is about 25 bar/m bed height at a superficial velocity of 3,000 cm/hour.

The results of the comparison are summarized in the following table, wherein:

B = bed volumes of cheese whey per hour.

KV = volume of the column bed in l.

h = column bed height in m.
d = column bed diameter in m.

Table

B	KV	S-Sepharose h	Fast Flow d	S-Sepharose h	Big Beads d
60	333	0.067	2.52*	0.5	0.92
90	222	0.044	2.52*	0.33	0.92
150	133	0.027	2.52	0.20	0.92
200	100	0.020	2.52	0.15	0.92*
300	67	0.013	2.52	0.10	0.92*
450	44	0.009	2.52	0.067	0.92

* Optimum working conditions

This table clearly shows that the exceptionally high superficial velocity in the case of the new type of resin offers major advantages with respect to the permissible dimensions of the columns (diameter, bed height) and with respect to the permissible cheese whey loads on the columns (important parameters for the economy of the process).

Example 4

This example was set up to determine the binding capacity and the yield.

A column having a diameter of 1.6 cm was packed with 20 ml S Sepharose Big Beads ion exchanger. The bed height was 10 cm. After equilibration of the resin with 0.025 M phosphate buffer pH 6.5, clarified cheese whey was pumped through the column at a rate of 150 bed volumes/hour in the first test and at a rate of 200 bed volumes/hour in the second test. The cheese whey contained, respectively, 36 and 40 mg/l lactoferrin and, respectively, 17 and 19 mg/l lactoperoxidase. The total throughput of cheese whey was 34 l (about 1,700 bed volumes) in the first test and 20 l (about 1,000 bed volumes) in the second test.

After washing of the column with buffer, lactoperoxidase and lactoferrin were separately eluted in the conventional manner with, respectively, 0.3 M NaCl in phosphate buffer and 0.8 M NaCl in phosphate buffer. The eluates obtained were analysed for LP and LF content by means of HPLC.

The results are summarized in the following table:

	Test 1 (150 bed volumes/hour)		Test 2 (200 bed volumes/hour)	
	LP	LF	LP	LF
Bound amount g/l resin	27	57	15	36
Yield % from the whey	85	94	89	91

Example 5

A 10 cm column of a pilot production chromatography system (Bioprocess System Pharmacia) was packed with SP Sepharose Big Beads ion exchanger. The diameter of the column was 10 cm, the bed height was 11 cm and the bed volume was 0.864 l.

After equilibration of the resin at room temperature with 0.025 M phosphate buffer pH 6.5, clarified cheese whey was pumped through the column for 3 hours and 35 minutes at a rate of flow of 180 liter/hour. The superficial velocity was 2,300 cm/hour. The liquid load in this case was 208 bed volumes/hour. The total throughput of cheese whey was 650 l.

After washing of the column with buffer solution, lactoperoxidase (LP) and lactoferrin (LF) were separately eluted with, respectively, 0.35 M NaCl in phosphate buffer and 1.0 M NaCl in phosphate buffer. The liquid flow rate during

washing and elution was 8.6 liter/hour (10 bed volumes/hour). Fig. 3 shows the elution diagram.

Thus, 2.8 l LP eluate and 2.2 l LF eluate were obtained. The LP content of the LP fraction was determined by means of the enzymatic method and via HPLC, being 4.6 g/l. The LP yield was 94% relative to the LP in the whey.

The LF of the LF fraction, determined by means of UV absorption (A280) and HPLC, was 9.0 g/l. The LF yield was 96% relative to the LF in the whey.

The LP eluate and the LF eluate were desalted by means of ultrafiltration/diafiltration with the Pellicon system (Millipore) equipped with polysulfone membranes having a cut-off value of 10 kD, and subsequently freeze-dried.

The purities of LP in the LP preparation and LF in the LF preparation, determined by means of HPLC, were 93% and 94%, respectively, calculated on total solids.

These results demonstrate that if this type of ion exchanger is used in columns on a pilot production scale, extremely high superficial velocities of the whey can be applied and that, further, very high yields and purities of the two bio-active proteins are obtained.

Example 6

Analogously to Example 5, a chromatography column having a diameter of 10 cm was packed with SP Sepharose Big Beads ion exchanger to a bed height of 4.8 cm.

The bed volume was 0.377 l.

After equilibration of the ion exchanger as described in Example 5, clarified cheese whey was pumped through the column at a flow rate of 513 bed volumes/hour (193 l/h). The superficial velocity was 2,460 cm/hour. A total amount of 250 l whey was passed through the column.

The LP and LF-charged column was washed and eluted as described in Example 5, with a liquid flow rate of 10 bed volumes/hour.

The obtained eluates with LP and LF, 1.5 l and 1.3 l, respectively, were analysed for the content of bio-active proteins in the conventional manner.
The LP fraction contained a total of 4.70 g LP and the yield from the whey was 82%. The LF fraction contained a total of 6.75 g LF and the yield from the whey was 90%

CLAIMS

1. A process for isolating bio-active proteins such as lactoperoxidase and lactoferrin from milk and/or milk derivatives, characterized by the steps of:
   a) adsorbing said proteins to a cation exchanger by passing the milk or the milk derivative over the cation exchanger at a high superficial velocity (more than 500 cm per hour) and a high liquid load (100-600 bed volumes per hour);
   b) eluting said proteins, separately or simultaneously, using one or more salt solutions, so as to form one or more eluates, optionally followed by
   c) drying of said eluates.

2. A process according to claim 1, characterized in that the cation exchanger is present as an adsorption bed in a column, the superficial velocity of the milk and/or milk derivatives being 2,000-3,000 cm per hour and a liquid load of 100 to 300 bed volumes per hour.

3. A process according to any one of the preceding claims, characterized in that the binding capacity of the ion exchanger is more than 10 g lactoperoxidase and more than 10 g lactoferrin per liter bed volume and more than 80% of the said proteins is extracted.

4. A process according to any one of the preceding claims, characterized in that the milk derivative is chosen from the group consisting of skim milk, evaporated skim milk, buttermilk, milk protein concentrate, cheese whey, casein whey, whey concentrate, desalted whey, desalted whey concentrate and whey-protein concentrates.

5. A process according to claims 1-4, characterized in that milk derivatives are first clarified by removing coarser contaminants.

6. A process according to any one of the preceding claims, characterized in that said proteins are eluted from the column, separately or simultaneously, with salt solutions having a concentration of 2.5 mol at most, more particularly 2 mol.

7. A process according to claim 6, characterized in that said eluates are dried through vacuum-drying, freeze-drying, spray-drying or roller-drying, which drying step may or may not be preceded by a desalting and/or concentrating step.

8. Lactoperoxidase, lactoferrin, or mixtures of these proteins obtained according to any one of the preceding claims.

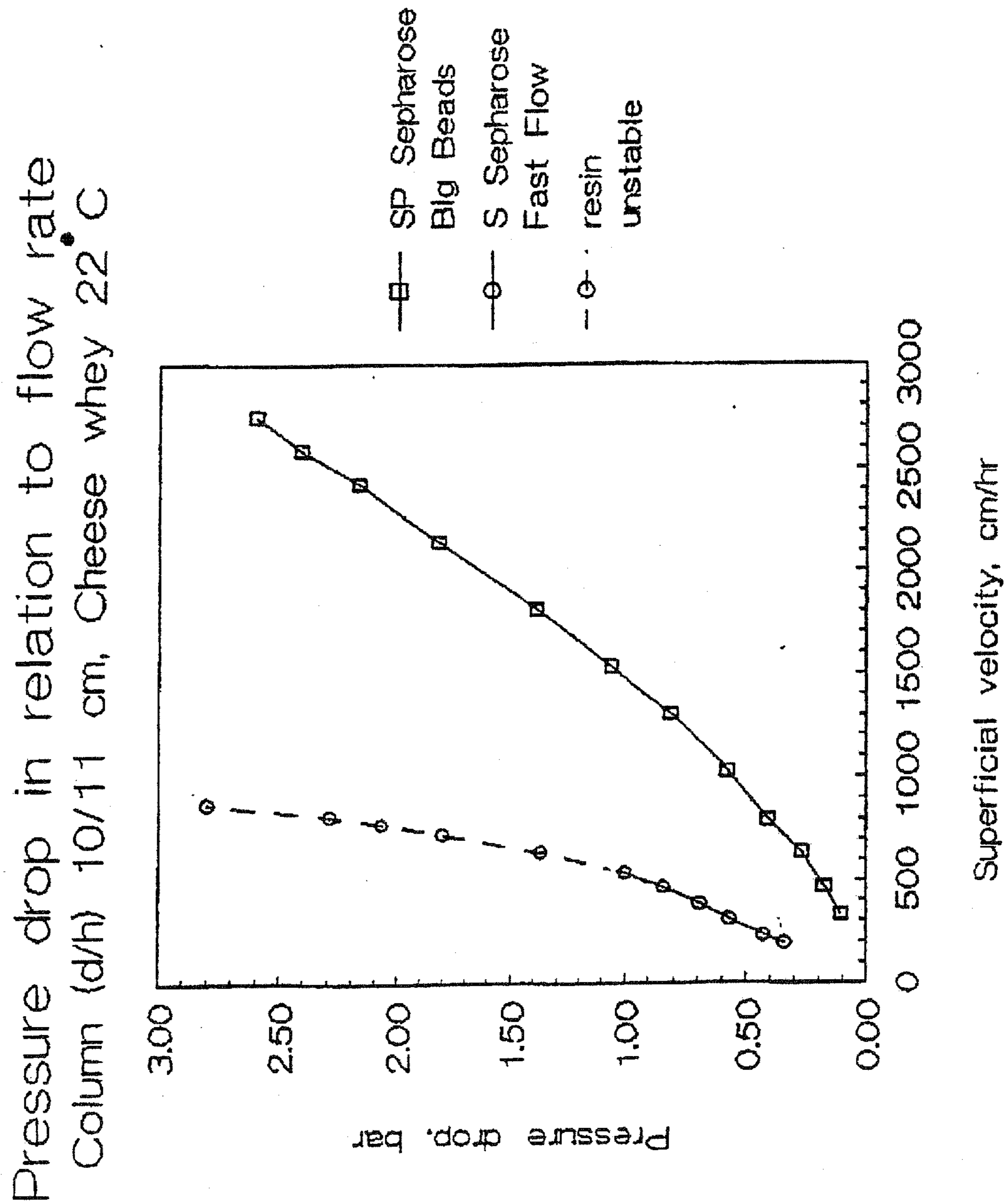
Pressure drop in relation to flow rate
Column (d/h) 10/11 cm, Cheese whey 22°C
SP Sepharose Big Beads
S Sepharose Fast Flow
resin unstable
Pressure drop, bar
0.00
0.50
1.00
1.50
2.00
2.50
3.00
0
500
1000
1500
2000
2500
3000
Superficial velocity, cm/hr

FIG. 1

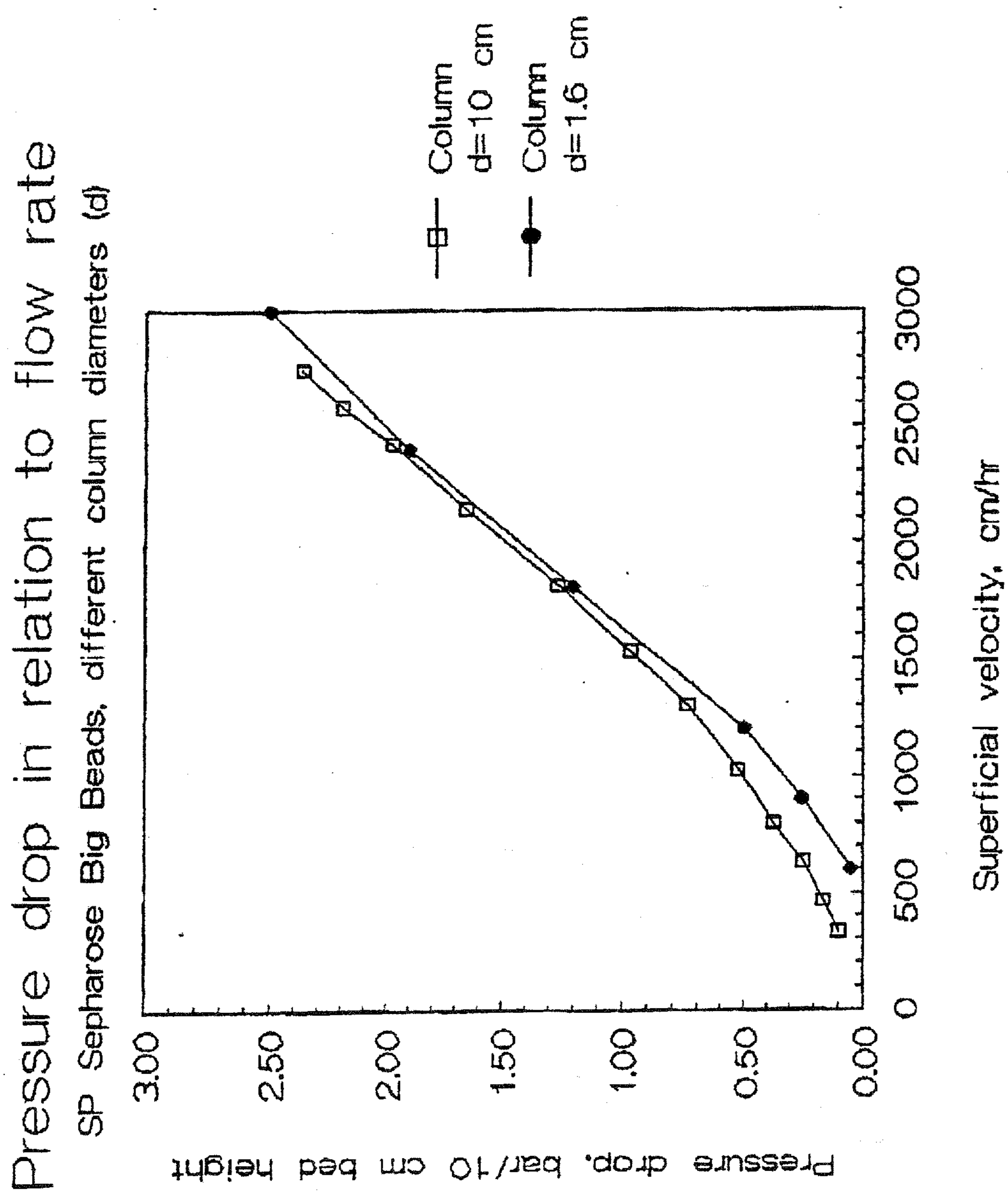
Pressure drop in relation to flow rate
SP Sepharose Big Beads, different column diameters (d)
Column d=10 cm
Column d=1.6 cm
Pressure drop, bar/10 cm bed height
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
500
1000
1500
2000
2500
3000
Superficial velocity, cm/hr

FIG. 2

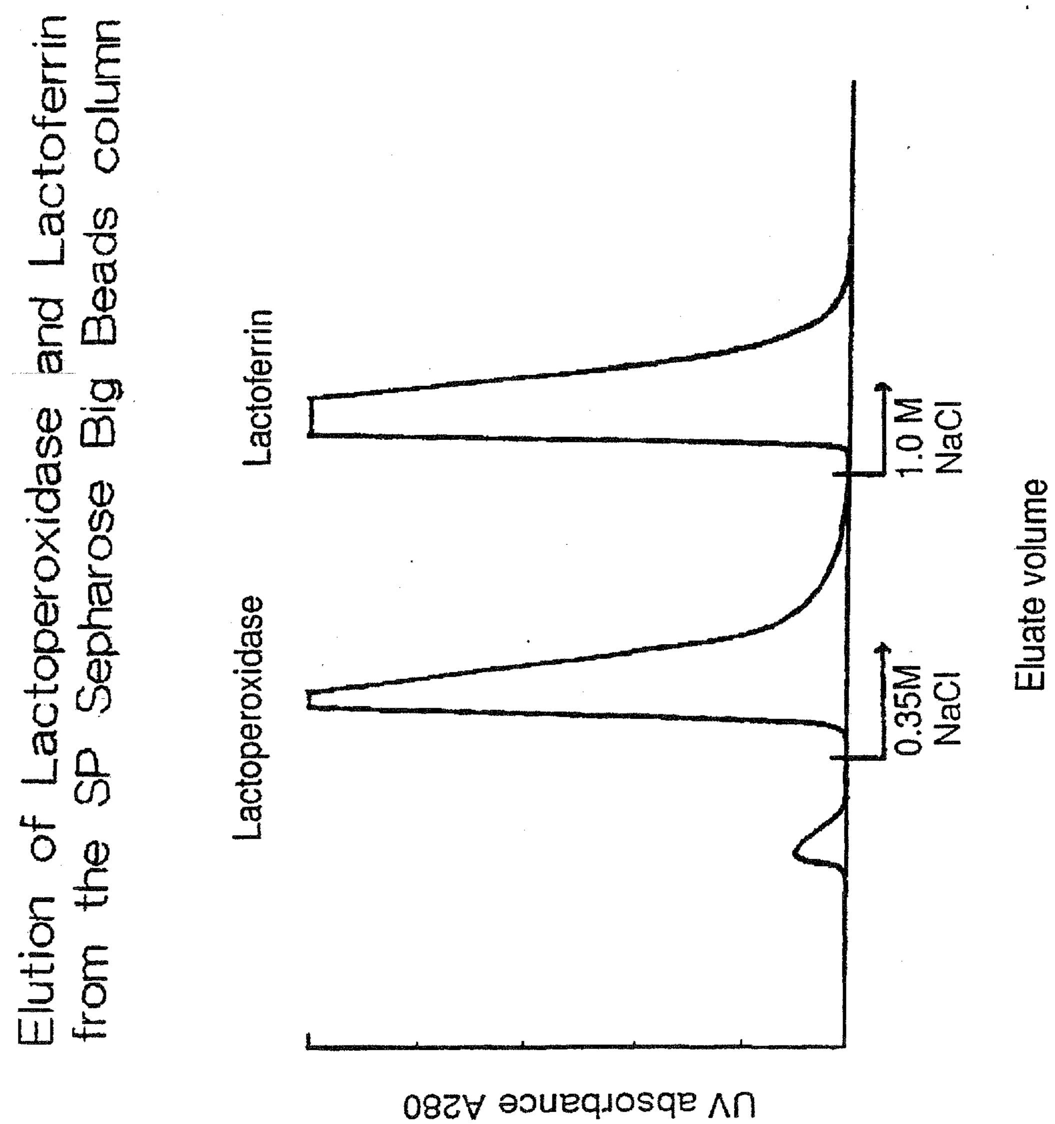
Elution of Lactoperoxidase and Lactoferrin
from the SP Sepharose Big Beads column
Lactoperoxidase
Lactoferrin
0.35M
NaCl
1.0 M
NaCl
Eluate volume
UV absorbance A280

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 93/00014

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]
According to International Patent Classification (IPC) or to both National Classification and IPC
Int.Cl. 5 A23J1/20; C07K3/22; C07K1/14; C07K15/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A23C ; A23J ; C07K ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	EP,A,0 418 704 (SNOW BRAND MILK PRODUCTS CO. LTD) 27 March 1991 cited in the application	1-8
Y	see column 2, line 34 - line 55; claims 1,2,5; examples 1,3 see column 3, line 16 - line 51 see column 4, line 25 - line 38 ---	2,6
X	EP,A,0 446 876 (SNOW BRAND MILK PRODUCTS & CO. LTD.) 18 September 1991 see page 3, line 53 - line 58; claims 1,4; examples 6,7 see page 4, line 17 - line 29 see page 5, line 4 - line 7 ---	1,2,4,5, 7,8

-/--

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

2

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
03 MAY 1993	24. 05. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	KANBIER D.T.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y A	EP,A,0 352 678 (SNOW BRAND MILK PRODUCTS) 31 January 1990 see column 3, line 19 - line 26; claims 1-4; figure 1B; examples 1-3 see column 7, line 29 - line 32	1,2,4,6 2,6 3
Y	BE,A,901 672 (OLEOFINA S.A.) 7 August 1985 see page 3, paragraph 1 -paragraph 4; claims 1,7-10,13; example 2 see page 4, paragraph 2 see page 6, paragraph 3 see page 7, paragraph 3 -paragraph 5 see page 7, paragraph 7 - page 8, paragraph 1	2,6
A	EP,A,0 284 525 (SOCIETE NATIONALE ELF AQUITAINE) 28 September 1988 see claims 1,2,5,6; figure; examples 1,3	1,4,6-8
A	GB,A,2 098 998 (SOCIETE NATIONALE ELF AQUITAINE) 1 December 1982 see page 2, right column, line 75 - line 80; claims 1,5,9-14,16; example 1	3
A	PATENT ABSTRACTS OF JAPAN vol. 014, no. 306 3 July 1990 & JP,A,21 04 246 (MORINAGA MILK IND. CO. LTD.) 17 April 1990 see abstract	1,4-8

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 9300014 SA 69694

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0418704	27-03-91	JP-A- 3109400	09-05-91
EP-A-0446876	18-09-91	JP-A- 4211100	03-08-92
		US-A- 5179197	12-01-93
EP-A-0352678	31-01-90	JP-A- 2138295	28-05-90
		US-A- 5087369	11-02-92
BE-A-901672	07-08-85	AU-B- 598186	21-06-90
		AU-A- 5248086	14-08-86
		CA-A- 1238633	28-06-88
		CH-A- 666692	15-08-88
		DE-A- 3603764	07-08-86
		FR-A,B 2576752	08-08-86
		GB-A,B 2171102	20-08-86
		JP-A- 61246198	01-11-86
		LU-A- 86252	09-06-86
		NL-A- 8600231	01-09-86
		SE-B- 468537	08-02-93
		SE-A- 8600423	08-08-86
		US-A- 4667018	19-05-87
EP-A-0284525	28-09-88	FR-A- 2613368	07-10-88
GB-A-2098998	01-12-82	FR-A- 2505615	19-11-82
		AU-B- 557353	18-12-86
		AU-A- 8372082	18-11-82
		BE-A- 893156	12-11-82
		CA-A- 1171723	31-07-84
		CH-A- 650905	30-08-85
		DE-A- 3218348	02-12-82
		JP-B- 3054118	19-08-91
		JP-A- 58028233	19-02-83
		NL-A- 8201947	01-12-82
		SE-B- 460669	06-11-89
		SE-A- 8203056	16-11-82
		US-A- 4436658	13-03-84

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82